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Lecanicillium uredinophilum sp. nov. associated with rust fungi from Korea

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Abstract — A novel species of *Lecanicillium* found on rust fungi is described. *Lecanicillium uredinophilum* sp. nov. is closely related to *L. longisporum* but differs morphologically in having longer phialides. Multiple gene sequence analysis supports its taxonomic position in the genus *Lecanicillium*.

Key words — *Cordycipitaceae*, fungicolous species, hyperparasitism, *Verticillium*-like hyphomycete

Introduction

The genus *Lecanicillium*, typified by *L. lecanii*, was established to accommodate entomogenous or fungicolous species that had been previously assigned to *Verticillium* sect. *Prostrata* (Gams & Zare 2001). Subsequently, Zare & Gams (2001) described 14 *Lecanicillium* species based on a combination of morphological characteristics and analysis of the internal transcribed spacer (ITS) rDNA sequence. Subsequently, nine additional taxa have been placed in *Lecanicillium* (Kope & Leal 2006, Zare & Gams 2008, Sukarno et al. 2009, Kaifuchi et al. 2013). Phylogenetically, the genus *Lecanicillium* is placed in *Cordycipitaceae* Kreisel ex G.H. Sung et al. (Sung et al. 2007). The genus is characterized by elongated conidia and simple phialides that are held in whorls or singly, arising from prostrate aerial hyphae. Sung et al. (2007) demonstrated that the genus *Lecanicillium* is not monophyletic but paraphyletic, a fact that was inferred from multigene phylogenetic analysis using the nuclear ribosomal

small and large subunits (SSU, LSU), translation elongation factor 1-α (TEF1), and the largest and second largest subunits of RNA polymerase II (RPB1, RPB2). More recently, Kouvelis et al. (2008) showed that phylogenetic analysis, using the three mitochondrial genes and the ITS region combined, placed some taxonomically uncertain isolates of *Verticillium* species into *Lecanicillium*.

During extensive surveys on the occurrence of hyperparasitic fungi attacking rusts in Korea, three isolates of a *Lecanicillium*-like fungus were obtained from the rust sori. In this study, these fungi are characterized based on their in vivo and in vitro morphology. Furthermore, the phylogenetic positions of the isolates have been investigated by sequence analysis of the combined genes SSU, LSU, TEF1, RPB1, and RPB2. As a result, these isolates are identified as representing a new species of *Lecanicillium*.

Materials & methods

Isolates

Three collections of rust infected with *Lecanicillium* were collected from several localities of Korea during 2008–2009. Single spore isolates from fresh materials were successfully obtained and grown on PDA at room temperature for 4 weeks in the dark. The isolates were deposited in the Korean Agricultural Culture Collection (KACC) of the National Academy of Agricultural Science, Wanju, Korea. Voucher specimens are housed in the Korea University herbarium, Seoul, Korea (KUS). For this study, ex-type strains and reference isolates of *Lecanicillium* species were gathered from the Centraalbureau voor Schimmelcultures (CBS, the Netherlands) and analyzed together with the Korean isolates. Detailed information on all the isolates used is shown in Table 1.

Morphological study

Fungal structures from air-dried specimens were mounted in 3% KOH solution, whereas those from monoconidial isolates were mounted in sterilized water. They were measured at magnifications of 400× and 1000×, using an eyepiece micrometer and a model BX51 microscope (Olympus, Tokyo, Japan). Microscopic images were photographed using an Axio imager microscope (Carl Zeiss, Göttingen, Germany).

Molecular phylogenetic analysis

DNA extraction, PCR amplification, and sequencing — For genomic DNA extraction, mycelial mats and conidia of the *Lecanicillium* isolates were harvested by scraping the surface of colonies that had been grown on PDA plates for one month. Total genomic DNA was prepared according to the method of Lee & Taylor (1990). PCR amplification was performed for the SSU rDNA using primers NS1 and NS4 (White et al. 1990), and for the LSU rDNA using primers LROR and LR7 (Vilgalys & Hester 1990). The PCRs for these two regions of rDNA were conducted as follows: an initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, extension for 2 min at 72°C, and a final extension for 10 min at 72°C. A fragment of the TEF1 gene was amplified using the primer pair EF1-983F

Table 1. *Lecanicillium* and *Simplicillium* sequences used in phylogenetic analyses.

Taxon	Isolate	GenBank Accession No.				
		SSU	LSU	TEF1	RPB1	RPB2
L. uredinophilum	KACC 44082	KM283758	KM283782	KM283806	KM283828	KM283848
	KACC 47756	KM283759	KM283783	KM283807	KM283829	KM283849
	KACC 44066	KM283760	KM283784	KM283808	KM283830	KM283850
L. acerosum	CBS 418.81^{T}	KM283762	KM283786	KM283810	KM283832	KM283852
L. antillanum	CBS 350.85^{T}	AF339585	AF339536	DQ522350	DQ522396	DQ522450
L. aphanocladii	CBS 797.84^{T}	KM283763	KM283787	KM283811	KM283833	KM283853
L. aranearum	CBS 726.73A	AF339586	AF339537	EF468781	EF468887	EF468934
L. attenuatum	CBS 402.78	AF339614	AF339565	EF468782	EF468888	EF468935
L. dimorphum	CBS 345.37	KM283764	KM283788	KM283812	KM283834	KM283854
	CBS 363.86^{T}	AF339608	AF339559	EF468784	EF468890	—
L. flavidum	CBS 300.70D	KM283765	KM283789	KM283813	—	KM283855
	CBS 342.80^{T}	KM283766	KM283790	KM283814	—	—
L. fungicola var. *aleophilum*	CBS 357.80^{T}	KM283767	KM283791	KM283815	KM283835	KM283856
L. fungicola var. *fungicola*	CBS 992.69^{T}	KM283768	KM283792	KM283816	—	KM283857

Table 1 concluded on p. 1000

Table 1, concluded

Taxon	Isolate	GenBank Accession No.				
		SSU	LSU	TEF1	RPB1	RPB2
L. fusisporum	CBS 162.70	KM283769	KM283793	KM283817	KM283836	KM283858
	CBS 164.70^{T}	AF339598	AF339549	EF468783	EF468889	—
L. lecanii	CBS 101247	KM283770	KM283794	DQ522359	KM283837	KM283859
	CBS 102067^{T}	KM283771	KM283795	KM283818	KM283838	KM283860
L. longisporum	CBS 102072	KM283772	KM283796	KM283819	KM283839	KM283861
	CBS 126.27^{T}	KM283773	KM283797	KM283820	KM283840	KM283862
L. muscarium	CBS 143.62^{T}	KM283774	KM283798	KM283821	KM283841	KM283863
L. pissodis	CBS 118231^{T}	KM283775	KM283799	KM283822	KM283842	KM283864
L. psalliotae	CBS 367.86	KM283776	KM283800	KM283823	—	—
L. saksenae	CBS 532.81^{T}	AF339609	AF339560	EF469067	EF469096	EF469112
Lecanicillium sp.	CBS 639.85	KM283777	KM283801	KM283824	KM283843	KM283865
L. tenuipes	CBS 309.85	KM283778	KM283802	DQ522341	KM283844	KM283866
L. wallacei	CBS 101237^{T}	AY184978	AY184967	EF469073	EF469102	EF469119
Simplicillium lanosoniveum	CBS 101267	AF339603	AF339554	DQ522357	DQ522405	DQ522463

and EF1-2218R (Rehner 2001). The PCR cycling conditions were as follows: an initial denaturation for 2 min at 96°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, extension for 30 s at 72°C, and a final extension for 7 min at 72°C. A region of the RPB1 gene was amplified with the primer pair CRPB1 and RPB1-Cr (Castlebury et al. 2004). The PCR cycling conditions used were as those described by Castlebury et al. (2004). A portion of the RPB2 gene was amplified with the primer pair fRPB2-5F and fRPB2-7cR (Liu et al. 1999). The PCR cycling conditions were as follows: an initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 10 s at 55°C, extension for 2 min at 72°C, and a final extension for 10 min at 72°C. The PCR amplicons were purified using MultiScreen HTSTM PCR filter plates (Millipore, Carrigtwohill, Cork, Ireland). Purified amplicons were directly sequenced on an ABI Prism™ 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with the same primer pairs used for the PCR amplification.

Sequence alignment and phylogenetic analysis — All resulting sequences were edited using DNAStar Lasergene Seqman™ and EditSeq™ software version 7.1.0 (DNAStar, Madison, WI, USA). Reference gene sequences were retrieved from GenBank. The RPB1 and PRB2 gene sequences were unobtainable for a few isolates and thus were treated as missing data in the phylogenetic analysis, and the areas of ambiguity in the alignments were excluded. Multiple sequence alignments were performed using MAFFT version 6.864 (Katoh et al. 2005) with the G-INS-i strategy. The alignments were manually adjusted, if needed. A phylogenetic tree was reconstructed from the Bayesian inference analysis. The model of DNA substitution was estimated with jModelTest version 0.1.1 (Posada 2008). The Bayesian inference analysis was performed using MrBayes version 3.0b4 (Ronquist & Huelsenbeck 2003). Four incrementally heated simultaneous Markov chains were run for one-million generations, with a tree saved at every 100th generation. The first 1000 trees generated from this analysis were discarded. A majority rule consensus tree showing all compatible partitions was obtained with the posterior probability (PP) for each group. Only PP values ≥50% were indicated in the resulting tree. Branch lengths were computed as the mean values over the trees sampled. The phylogenetic trees were viewed with TreeViewX version 0.5.0 (Page 1996). A sequence of *Simplicillium lanosoniveum* (J.F.H. Beyma) Zare & W. Gams (CBS 101267) served as outgroup for the multigene phylogenetic analysis.

Results

Molecular phylogenetic analysis

The resulting alignment included the sequences of the five genes from 27 *Lecanicillium* isolates and one outgroup isolate. The alignment consisted of 3999 characters (including alignment gaps) that comprised 952 characters of SSU, 759 of LSU, 871 of TEF1, 522 of RPB1, and 895 of RPB2. A majority rule consensus tree (Fig. 2) was generated from the Bayesian analysis, using the GTR + Γ + I model selected by jModelTest. The three Korean isolates found on uredinia of the rust fungi *Pucciniastrum agrimoniae* and *Coleosporium* sp. clustered into a separate clade. The three isolates (KACC 47756, KACC 44066, and KACC 44082), forming a distinct group (PP = 1.00), were closely related

with three *Lecanicillium* species, *L. muscarium* (Petch) Zare & W. Gams, *L. pissodis* Kope & I. Leal, and *L. attenuatum* Zare & W. Gams, within a strongly supported cluster (PP = 1.00).

Taxonomy

Lecanicillium uredinophilum M.J. Park, S.B. Hong & H.D. Shin, **sp. nov.** Fig. 1

MycoBank MB 814832

Differs from *Lecanicillium longisporum* by its longer phialides.

Type: Korea, Gangwon Province, Hongcheon, Seongdong-ri, 37°44′41″N 127°52′01″E, on uredinia of *Pucciniastrum agrimoniae* (Dietel) Tranzschel growing on *Agrimonia pilosa* Ledeb. (*Rosaceae*), 26 October 2008, collected and isolated by M.J. Park and H.D. Shin (**Holotype**, KUS-F23890; ex-type culture, KACC 44082; GenBank KM283758, KM283782, KM283806, KM283828, KM283848).

Etymology: The epithet refers to the uredinicolous habit of this fungus.

In vivo: Colonies on rust sori, loose or floccose, white. Mycelium partly immersed in rust sori but mostly superficial. Phialides rather long, 15–50 × 1–3 µm, hyaline, tapering towards the apex, arising from prostrate hyphae, solitary or up to 3–4 per node; secondarily produced phialides present. Conidia 3–6 × 1.5–3 µm, hyaline, cylindric to oblong or narrowly ellipsoid, aseptate.

In vitro: Colonies attaining 25–30 mm diameter in 10 d on PDA, white to cream colored, velvety to cottony; margin entire; reverse cream colored. Phialides 20–60 × 1–2.5(–3) µm, produced singly or in whorls of up to 3–4(–5) on prostrate hyphae. Conidia 3–9 × 1.8–3 µm, cylindric, oblong, or ellipsoid, mostly aseptate, rarely uniseptate.

Additional specimens examined:

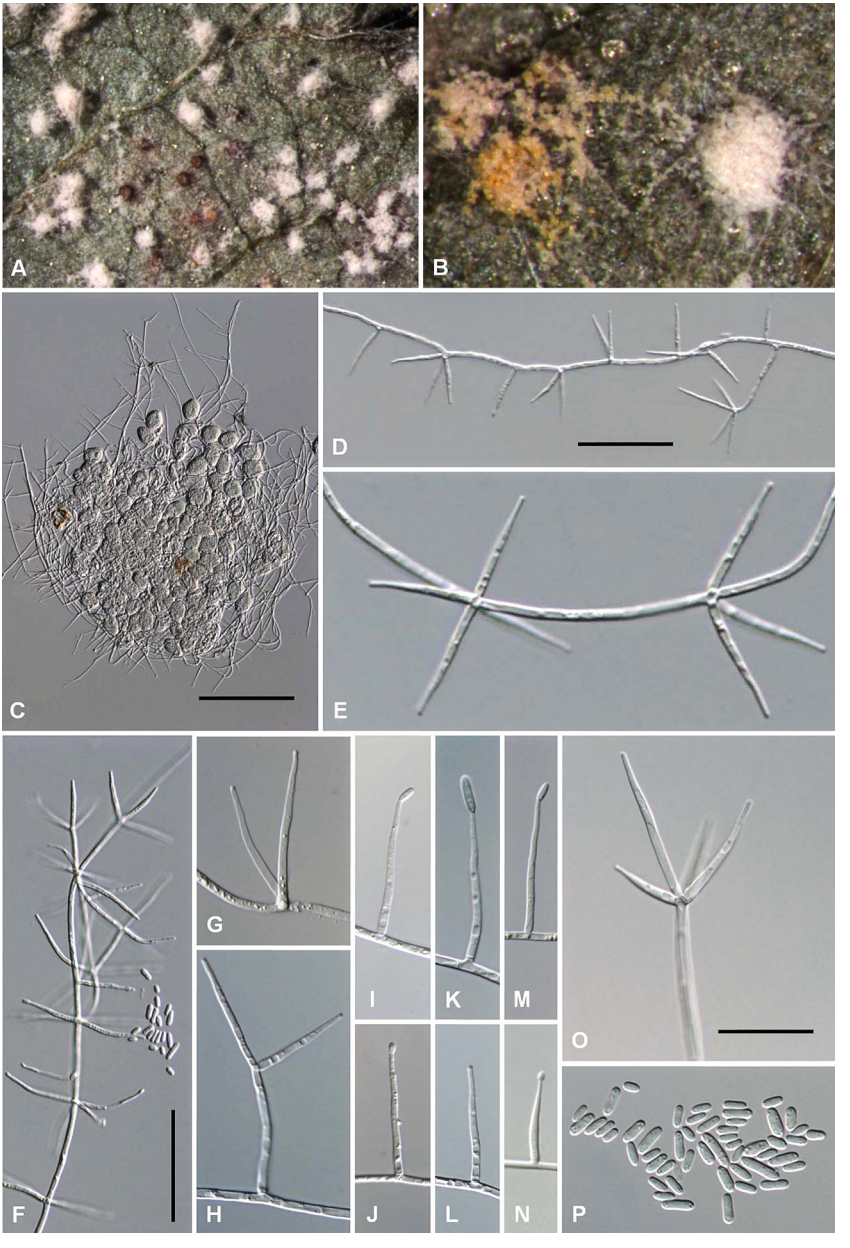
On uredinia of *Coleosporium* sp. growing on *Aster* sp. (*Asteraceae*): **KOREA**, **Gangwon Province**, Pyeongchang, Korea Botanic Garden, 37°42′24″N 128°36′49″E, 9 October 2008, collected and isolated by M.J. Park and H.D. Shin (KUS-F23782; culture, KACC 44066).

On uredosori of *Pucciniastrum agrimoniae* growing on *Agrimonia pilosa*: **KOREA**, **North Chungcheong Province**, Boeun, Okhwa recreational forest, 36°35′52″N 127°41′48″E, 2 August 2009, collected and isolated by M.J. Park (KUS-F24383; culture, KACC 47756).

Discussion

Three Korean fungal isolates found as rust hyperparasites were shown to represent a new species, *Lecanicillium uredinophilum*. The morphology of

Fig. 1. *Lecanicillium uredinophilum*. A: Colonies overgrowing uredinia of *Pucciniastrum agrimoniae* on *Agrimonia pilosa*. B: Non-hyperparasitized (left) and hyperparasitized (right) uredinia. C: Rust propagules densely intertwined with hyphae of *L. uredinophilum*. D–G, O: Verticillate phialides. H: Branched phialide. I–N: Solitary phialides. P: Conidia. Scale bars: C = 100 µm, D, F = 50 µm, E, G–P = 20 µm.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L. uredinophilum resembles that of *L. longisporum* (Petch) Zare & W. Gams, which differs by its shorter phialides (20–40 µm; Zare & Gams 2001). Based on their ITS rDNA (not presented in this study) and multigene phylogenies, the three *L. uredinophilum* isolates form a well-supported distinct clade that is distant from other *Lecanicillium* taxa. Morphological and phylogenetic analyses show that the isolates represent a previously unknown species, here described as *L. uredinophilum*.

Although the main hosts of *Lecanicillium* species are known to be insects and mushrooms (Zare & Gams 2001), the present investigation indicates that rust fungi are another major ecological substrate for *Lecanicillium* species. The discovery of *Lecanicillium* species associated with rust sori suggests that more species of *Lecanicillium* and *Verticillium*-like fungi remain to be discovered as hyperparasites of rusts worldwide.

Acknowledgments

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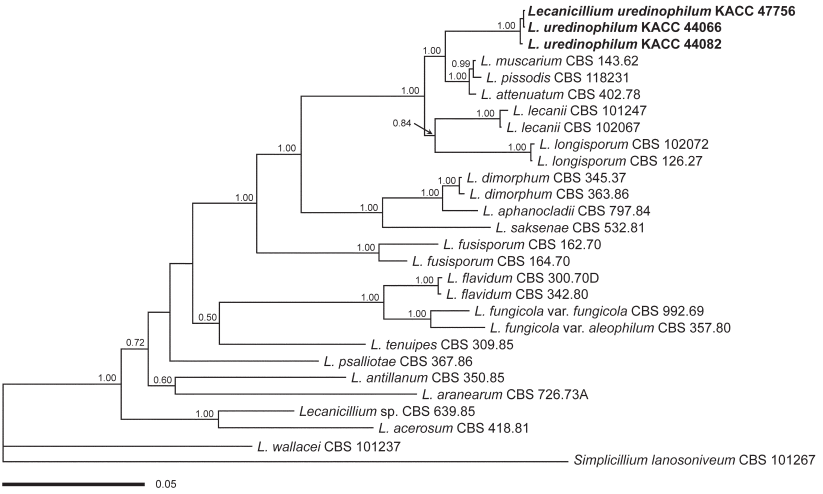
Lecanicillium uredinophilum KACC 47756
L. uredinophilum KACC 44066
L. uredinophilum KACC 44082
L. muscarium CBS 143.62
L. pissodis CBS 118231
L. attenuatum CBS 402.78
L. lecanii CBS 101247
L. lecanii CBS 102067
L. longisporum CBS 102072
L. longisporum CBS 126.27
L. dimorphum CBS 345.37
L. dimorphum CBS 363.86
L. aphanocladii CBS 797.84
L. saksenae CBS 532.81
L. fusisporum CBS 162.70
L. fusisporum CBS 164.70
L. flavidum CBS 300.70D
L. flavidum CBS 342.80
L. fungicola var. fungicola CBS 992.69
L. fungicola var. aleophilum CBS 357.80
L. tenuipes CBS 309.85
L. psalliotae CBS 367.86
L. antillanum CBS 350.85
L. aranearum CBS 726.73A
Lecanicillium sp. CBS 639.85
L. acerosum CBS 418.81
L. wallacei CBS 101237
Simplicillium lanosoniveum CBS 101267
0.05

Fig. 2. Phylogenetic relationship among taxa belonging to the genus *Lecanicillium* based on Bayesian analysis of the combined SSU, LSU, TEF1, RPB1, and RPB2 sequences. Isolates obtained from this study are in bold. The numbers at the nodes indicate the Bayesian inference posterior probability (BI PP) values. Only PP values ≥50% are shown. The number of nucleotide changes between taxa is represented by branch length and the scale bar indicates the number of nucleotide substitutions per site.

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